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Transport of D-glucose across cultured stratified cell layer of human oral mucosal cells

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Abstract

To evaluate the role of several specialized mechanisms for D-glucose transport in human oral mucosa, a cultured stratified cell layer derived from human oral mucosa was employed. Although this culture system has been used for reconstructive surgery, we, for the first time, tried to apply this system to the evaluation of nutrients and drug transport. Cell number and transepithelial electrical resistance (TEER) reached steady state 7–8 days after inoculation on the Transwell and TEER values at steady state were 130–140 Ω cm^2, which was higher or lower than that of small intestine or Caco-2 cells, respectively. The transport studies were carried out using the cultured epithelium on the Transwell. The transport of D-glucose across the cultured stratified layer of oral epithelial cells was much more extensive than L-glucose, and was inhibited by 2-deoxy-D-glucose, a substrate of facilitative glucose transporters, and α-methyl-D-glucoside, a specific substrate of a Na^+/glucose cotransporter (SGLT1). The results indicate that the sugar transporters function not only to take up D-glucose by the epithelial cells but also to transport the sugar across the stratified epithelial layer.

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Introduction

Generally, the mechanism of drug absorption from the oral cavity is passive diffusion following the pH-partition hypothesis and the lipoid theory. Some nutrients, however, have been shown to be absorbed by specialized transport mechanisms from the human oral cavity, using a buccal absorption test method (Evered et al 1980; Evered & Vadgama 1981; Evered & Mallet 1983; Hunjen & Evered 1985). Details of these transporters present in oral mucosal cells have not been clarified yet. In our previous study, the site specificity of the specialized transport system for D-glucose in the human oral cavity was demonstrated using newly designed perfusion cells: the specialized transport system for D-glucose was dominant in the dorsum of human tongue (Kurosaki et al 1998). Further, the uptake of glucose by epithelial cells isolated from human oral mucosa was examined to clarify the transport systems. The uptake of D-glucose was much larger in cells of the dorsum of tongue than in buccal cells. Western blotting analysis suggested that SGLT1 and GLUTs1–3 are expressed in both epithelial cells but inhibition studies with several sugar analogues suggested the larger contribution of facilitative transporters than Na^+/glucose cotransporter to D-glucose uptake by the epithelial cells of human oral mucosa (Oyama et al 1999). However, the question still remains whether the sugar transporters function only to take up the substrates

by the epithelial cells or also to transport them across the stratified epithelial layer. In this study, to answer this question, a cultured stratified cell layer of human oral mucosal cells was employed to examine the transport of glucose. The cultured stratified cell layer was developed by Rheinwald & Green (1975) and is being applied to reconstructive surgery (Tsai et al 1997). We tried to evaluate the contribution of these specialized mechanisms to the transport of D-glucose across the stratified cell layer.

Materials and Methods

Materials

^{14}C-D-Glucose, ^{3}H-L-glucose and ^{3}H-inulin were purchased from Amersham International (Buckinghamshire, UK). A liquid scintillator, Clear-Sol II, was purchased from Nacalai Tesque (Kyoto, Japan). D-Glucose (Ishizu Pharmaceutical Co., Osaka, Japan), L-glucose (Tokyo Kasei Kogyo Co., Tokyo, Japan) and 2-deoxy-D-glucose (Sigma Chemical Co., St Louis, MO) were used as supplied. Dulbecco's modified Eagle's medium (DMEM), Ham's F-12 medium and trypsin were purchased from Gibco Laboratories Life Technologies, Inc. (Grand Island, NY). Fetal bovine serum (FBS), penicillin G, cholera toxin, hydrocortisone, triiodothyronine, insulin (Sigma Chemical Co.) and dispase II (Boehringer Mannheim GmbH, Mannheim, Germany) were used as supplied. Mitomycin C (Kyowa Hakko Kogyo Co., Tokyo), kanamycin (Meiji Seika Co., Tokyo), amphotericin B (Fungizone, Bristol-Myers Squibb K.K., Tokyo) and human recombinant epidermal growth factor (rhEGF, Wakunaga Pharmaceutical Co., Hiroshima, Japan) were gifts from each company. Collagen-coated Snapwell (0.4 μm pore size, 1 cm^2 growth area) was purchased from Corning Costar Japan, Tokyo. All other chemicals were of reagent grade and commercially available.

Cell culture

Human oral mucosal epithelial cells were cultured according to the method of Hibino et al (1996). Alveolar gingiva, which was superfluous tissue debrided during surgery, was obtained from patients treated in the Department of Oral and Maxillofacial Surgery II of Okayama University Dental School Hospital. Our investigations were performed after approval by our local ethical committee at Okayama University and in accordance with the Declaration of Helsinki. Written informed consent was obtained from each patient before surgery.

Before epithelial cells were inoculated, the 3T3 cells used as the feeder layer were treated with 4 μg mL^{-1} mitomycin C in DMEM without serum for 2 h at 37°C, rinsed with phosphate-buffered saline solution (PBS) to remove mitomycin C, and cultured for 24 h in DMEM (13.5 g L^{-1}) containing 10% FBS. The 3T3 cells were inoculated onto dishes at a density of 2×10^4 cells/cm^2. The tissue segment, of which the submucosal tissue was removed with scissors, was cut into small pieces. To sterilize them, the tissue pieces were immersed twice in PBS containing 1000 IU mL^{-1} penicillin G, 1 mg mL^{-1} kanamycin and 2.5 μg mL^{-1} amphotericin B for 30 min at 37°C, and then immersed in the four-fold concentrated DMEM containing 0.8 IU mL^{-1} dispase II for 16 h at 4°C. Only the epithelium was treated with 0.25% trypsin solution for 30 min at room temperature to separate the cells. The specimens were washed with DMEM containing 10% FBS to remove enzymes and were stirred in DMEM containing 10% FBS for 30 min. The suspension was filtered through nylon gauze (50 μm) and the filtrate was centrifuged twice for 5 min at 1500 rev min^{-1}. The cell pellet was resuspended in culture medium (DMEM–Ham's F-12 medium, 3:1) supplemented with 5% FBS, 5 μg mL^{-1} insulin, 5 μg mL^{-1} transferrin, 2×10^{-9} M triiodothyronine, 1×10^{-9} M cholera toxin, 0.4 μg mL^{-1} hydrocortisone, 100 IU mL^{-1} penicillin G, 0.1 mg mL^{-1} kanamycin and 0.25 μg mL^{-1} amphotericin B, and was inoculated on the surface of the feeder layer at a density of 1×10^5 cells/cm^2 and kept in 5% CO_2. The medium was changed every 2 days. After the first change of medium, rhEGF was added to the medium at 10 ng mL^{-1}. In this study, the stratified cell cultures of five different passages or origins were used. The CV value for the interpassage variance was 44.6% in terms of D-glucose transport in 5 min and that for the intrapassage variance was ranged between 1.7 and 18.6%. Throughout all the experiments, cell cultures of passage number 3–5, and on 8 or 9 day after inoculation, were employed.

Cell proliferation

For cell counting, cultured cells were dispersed with 0.1% trypsin and suspended on each counting day. The cell number was counted with a haemocytometer and the total counts were compared. To obtain the number of epithelial cells, the number of resting 3T3 cells was subtracted from the total cell number. Cell counting was

repeated at least 6 times to obtain a more credible number.

Measurement of transepithelial electrical resistance (TEER)

To measure TEER, Snapwell on which cells were seeded was placed into a diffusion chamber (Corning Costar Japan) and this chamber system was maintained at 37°C. Both sides of the epithelial cells were bathed with 6 mL of incubation buffer consisting of (mM): 80 mannitol, 20 NaCl, 3 Tris chloride (pH 7.4), 1 K_2HPO_4, 1 $MgCl_2$, 6.8 $CaCl_2$ and 100 bovine serum albumin (Randles & Kimmich 1978). The cultured cells on Snapwell was short-circuited to zero transepithelial potential difference by a short-circuit current amplifier (CEZ-9000, Nihon Kohden Co. Tokyo). After pre-incubation for 25 min to stabilize the electrical condition of the cells, the TEER was determined following Ohm's law. The actual value of TEER across the stratified cell layer was obtained by subtracting the TEER value derived from filter of Snapwell and incubation buffer, $71.2 \pm 3.7\ \Omega\ cm^2$, from the measured value of TEER. It was also recognized that TEER value was almost constant during transport study for 5 min.

Transport study

After removal of culture medium from the confluent cell layer on the collagen-coated Snapwell, both the apical and basolateral sides were washed with the incubation buffer. Then, the cell layer was pre-incubated in the incubation buffer at 37°C for 30 min. The volume of incubation buffer was 0.5 mL and 2.0 mL in the apical and basolateral sides, respectively. The transport experiment was started by changing the incubation solution on the apical side to the solution containing radioactive substrates. Solution on the basolateral side (100 μL) was periodically sampled out and immediately replaced with the same volume of fresh incubation solution. The amounts transported were calculated as a function of time. All the transport experiments were performed at 37°C.

After finishing the transport experiment, the solution on the apical side was removed and the cell layer was washed very quickly. Then, the amount of sugars taken up by the cell was also determined. The sugar present in the intercellular medium was subtracted from the total amount associated with cells using ^{3}H-inulin as an extracellular marker according to a previous report (Yamazaki et al 1992). Since inulin cannot penetrate into cells, the apparent uptake of inulin should be attributed to inulin in the intercellular water, of which the concentration can be assumed to be equivalent to its concentration in the incubation buffer on the apical side. Therefore, the volume of intercellular water can be calculated by the following equation:

$$V_{intercellular} = \frac{A_{inulin}}{C_{inulin} \cdot A_{protein}} \quad (1)$$

where $V_{intercellular}$, A_{inulin}, C_{inulin} and $A_{protein}$ are the volume of intercellular water, the uptake amount of inulin, the concentration of inulin in apical solution and protein content of cells used for each transport study of ^{14}C-D-glucose, respectively. The averaged volume of the intercellular water was $3.79 \pm 0.79\ \mu L\ (mg\ protein)^{-1}$.

Inhibition study

To clarify the mechanisms of D-glucose transport across the stratified cell layer, inhibition studies were performed according to the procedure of the transport study described above. A transport inhibitor or a sugar analogue was dissolved in the incubation buffer containing ^{14}C-D-glucose for the apical side. For the Na^+-free study, Na^+ was replaced with choline$^+$ for the preparation of the incubation buffer.

Analytical methods

For transport studies, radioactivity was measured by a Beckman LS-232 liquid scintillation counter (Beckman Coulter, Inc., Fullerton, CA) using a liquid scintillator, Clear-Sol II. For measuring cellular uptake, the stratified cells detached from Snapwell were solubilized by adding 0.25 mL of 1 M NaOH and left for 30 min at room temperature. Then, the solution was neutralized with 0.25 mL of 1 M HCl before adding the liquid scintillator. Protein was determined by the method of Lowry et al (1951).

Statistical analysis

Analysis of valiance was performed to test the statistical significance of differences among groups. Statistical significance in the means was evaluated using Student's *t*-test or Dunnett's test. Results are expressed as mean ± s.d. of more than three experiments.

Results

Figure 1 shows the total cell number of cultured mucosal cells. The cells were proliferating from day 3 to day 8. Phase-contrast microscopic observation showed that mucosal cells became a confluent monolayer on the third day. The cells started to form colonies on the monolayer within 4 days and appeared to eliminate the 3T3 cells. On day 6, most of the 3T3 cells were eliminated. The stratification was clearly observed on day 8. As shown in Figure 2, the change in TEER well reflected the change in the cell number. Judging from these results, transport studies were carried out using the cell layer of day 8 or 9.

Figure 3 shows the time course of glucose transport across the cultured stratified cell layer of human oral mucosal cells at the initial concentration of 0.5 mM. Although significant transport of L-glucose was observed, the transport of D-glucose was much greater, suggesting that some transporter for D-glucose should function to transport the sugar across the stratified cell layer. Figure 4 shows the concentration dependency of the transport across the stratified cell layer in 5 min. At all the concentrations examined, the transport of D-

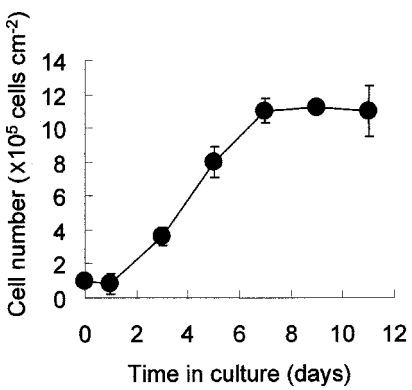

Figure 1 Total cell counts of cultured stratified cell layer of human oral mucosal cells. Results are expressed as the mean±s.d. of three experiments.

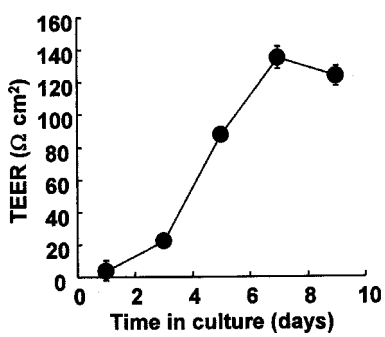

Figure 2 Transepithelial electrical resistance (TEER) of cultured stratified cell layer of human oral mucosal cells at different stages of confluence. Results are expressed as the mean±s.d. of three experiments.

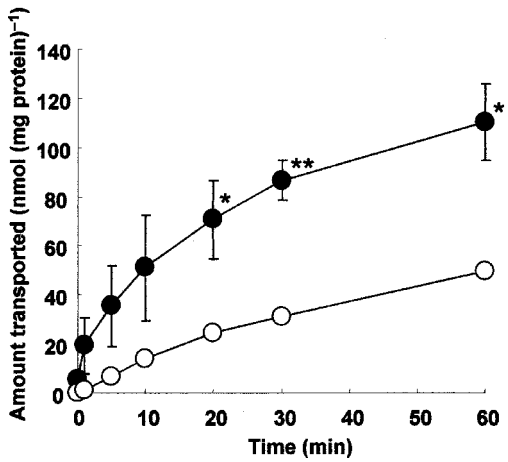

Figure 3 Time course of D-glucose (●) and L-glucose (○) transport across cultured stratified cell layer of human oral mucosal cells. Results are expressed as the mean±s.d. of three experiments. Bars showing s.d. for L-glucose are hidden behind the symbols. Initial concentrations of D- and L-glucose were 0.5 mM. $*P < 0.05$; $**P < 0.01$, compared with L-glucose.

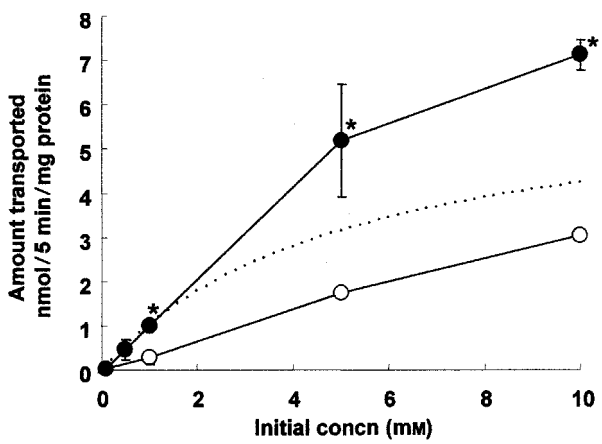

Figure 4 Concentration dependency of D-glucose (●) and L-glucose (○) transport across cultured stratified cell layer of human oral mucosal cells. Results are expressed as the mean±s.d. of three experiments. Bars showing s.d. for L-glucose are hidden behind the symbols. The dotted line was drawn using parameters obtained by fitting the differences in the transport rates between D-glucose and L-glucose to the Michaelis–Menten equation. $*P < 0.05$, compared with L-glucose.

Table 1 Effect of special conditions, transport inhibitors and sugar analogues on transport of glucose across stratified cell layer of human oral mucosal cells.

Condition or additive	Amount transported in 5 min (% of control)	Amount in cells at 5 min (% of control)
Control (0.5 mM D-glucose)	100±18.6	100±15.6
with 0.5 mM *p*-chloromercuribenzoic acid	64.6±4.2**	66.9±11.4**
with 1 mM 2,4-dinitrophenol	63.8±7.3**	55.1±3.2**
with 1 mM phlorizin	51.4±3.9**	64.0±6.9**
Na^+-free	58.4±5.2**	56.7±6.2**
with 20 mM α-methyl-D-glucoside	63.7±6.7**	56.7±12.4**
with 20 mM 2-deoxy-D-glucose	48.7±14.0**	45.5±9.0**
L-Glucose transport (0.5 mM)	39.5±3.8**	4.5±1.9**

Results are expressed as the mean±s.d. of four experiments. Control values of the amount transported in 5 min and the amount remaining in cells at 5 min were 26.97±5.03 nmol/5 min/mg protein and 10.62±1.66 nmol (mg protein)$^{-1}$, respectively. **$P < 0.01$, compared with control values.

glucose was greater than L-glucose. While the transport rate of L-glucose was in proportion to the initial concentration, a saturable tendency in the transport rate of D-glucose was observed. The transport of D-glucose via the carrier-mediated system is shown by the dotted line, which was drawn using the parameters obtained by fitting the differences of the transport rates between D-glucose and L-glucose to the Michaelis–Menten equation. The values of K_m (Michaelis constant) and V_{max} (the maximum transport rate) thus obtained were 5.3±3.9 mM and 6.5±2.1 mol/5 min/mg protein, respectively.

The effect of Na^+-free medium and transport inhibitors on the transport of D-glucose across the stratified cell layer was examined and the results are shown in Table 1. The transport of D-glucose was significantly inhibited by 0.5 mM *p*-chloromercuribenzoic acid, suggesting the contribution of a carrier protein to the transport process. In addition, D-glucose transport was reduced in the Na^+-free medium and was inhibited by 2,4-dinitrophenol and phlorizin, suggesting that an Na^+-dependent D-glucose transport system operates at least partly in the transport process. To further clarify the carrier system involved in D-glucose transport across the stratified cell layer, the effect of two specific sugars (20 mM) on D-glucose transport (0.5 mM) was examined and the results are also shown in Table 1. Both α-methyl-D-glucoside, a specific substrate of SGLT1, and 2-deoxy-D-glucose, a substrate of facilitative transporters, significantly inhibited D-glucose transport, the inhibitory activity of 2-deoxy-D-glucose being larger than that of α-methyl-D-glucoside. Similarly, the amount of D-glucose taken up in the mucosal cells at 5 min was reduced more extensively in the presence of 2-deoxy-D-glucose than in the presence of α-methyl-D-glucoside.

Discussion

D-Glucose is one of the nutrients that can be absorbed from the oral cavity by a carrier-mediated mechanism (Manning & Evered 1976). In our previous study, we showed, using perfusion cells for human oral mucosa, that the carrier-mediated absorption of D-glucose is predominant in the dorsum of tongue (Kurosaki et al 1998). Furthermore, we demonstrated, using the isolated cells of human oral mucosa, buccal epithelium and the dorsum of tongue, that D-glucose is taken up not only by the Na^+/glucose cotransporter but also by facilitative glucose transporters. The expression of SGLT1, GLUT1, GLUT2 and GLUT3 in the epithelial cells of human oral mucosa was also confirmed by Western blotting analysis (Oyama et al 1999). However, it remained to be clarified whether the sugar transporters function only to take up the substrates by the epithelial cells or also to transport them across the stratified epithelial layer. To answer this question, in this study, we examined the transport characteristics of glucose across the cultured stratified cell layer of human oral mucosal cells.

Cell culture was performed according to the method of Hibino et al (1996). This method is well established, and the mucosal cell sheet thus obtained is used for reconstructive surgery (Tsai et al 1997). As shown previously (Hibino et al 1996), the mucosal sheets cultured by this method have approximately 3–5 layers microscopically, and the mucosal cells in the basal layer

and upper portion have nuclei. In this study, we showed the usefulness of the stratified cells for transport study. However, it was also suggested that some improvement was needed for this system to obtain more steady evaluation, because there was a rather high interpassage variance.

The TEER value was higher or lower than that of small intestine or Caco-2 cells, respectively (Tanaka et al 1995). Jacobsen et al (1995) and Nielsen & Rassing (1999) reported the TEER values of TR146 cells, which are derived from a human neck metastasis originating from a buccal carcinoma and form the stratified epithelial layer. According to the report by Jacobsen et al (1995), the maximal value of TEER for this cell line was $68.2 \pm 2.3\ \Omega\ cm^2$ and another value, $272 \pm 132\ \Omega\ cm^2$, was reported later (Nielsen & Rassing 1999). Although the latter value shows a large variance of TEER, our maximal value of TEER, $134.8 \pm 7.0\ \Omega\ cm^2$, is in the middle of the two values reported so far. TEER measurement suggested that the cell sheet is not so tight in spite of its stratified structure compared with Caco-2 and porcine buccal sheet ($872 \pm 378\ \Omega\ cm^2$; Nielsen & Rassing 1999). This might be partly due to lack of tight junctions in the mucosal cells, although the present stratified cells have a smaller number of desmosomes and looser intercellular junctions (Hata et al 1995).

The transport of D-glucose across the stratified cell layer of oral mucosal cells was much greater than that of L-glucose (Figure 3), while the difference was less than that in cell uptake (Oyama et al 1999), reflecting the relatively leaky structure. Furthermore, the transport of D-glucose, but not L-glucose, showed saturable tendency (Figure 4). These results strongly indicate that D-glucose transporters function not only to take up sugar by epithelial cells but also to transport it across the stratified epithelial layer. That is to say, the sugar transporters would function to absorb D-glucose from the oral cavity to the blood circulation.

The values of K_m and V_{max} for the carrier-mediated transport system of D-glucose were 5.3 ± 3.9 mM and 6.5 ± 2.1 nmol/5 min/mg protein, respectively. This K_m value is a pooled parameter of parallel Michaelis–Menten equations, and was much higher than the one obtained in the study on the uptake by isolated oral mucosal cells (Oyama et al 1999), but similar to the one obtained in the buccal absorption test (Kurosaki et al 1998). The difference might be caused by the complex contribution of glucose transporters to the uptake into cells and the efflux from the cells in the transport process.

To further investigate the carrier-mediated system in the transport of D-glucose across the stratified cell layer, inhibition studies were performed (Table 1). D-Glucose transport was inhibited by *p*-chloromercuribenzoic acid, suggesting that the SH group of the transporters might be involved in the transport process. The Na^+-dependency of the D-glucose transport and its inhibition by 2,4-dinitrophenol and phlorizin suggest that an Na^+-dependent D-glucose transporter, SGLT1, would contribute to D-glucose transport across the stratified cell layer. The amount of L-glucose taken up in the mucosal cells at the end of the transport experiment was markedly smaller than D-glucose. The amount of L-glucose taken up into the cells (4.5% of D-glucose) was much less than the amount transported (39.5% of D-glucose) (Table 1), which means that a significant amount could be transported through the relatively leaky intercellular space. However, D-glucose transport was significantly greater than L-glucose transport (Figures 3 and 4) which should be equivalent to the transport of D-glucose via passive diffusion including transport through the intercellular space. Furthermore, the amount of D-glucose taken up by cells in 5 min did reduce under several inhibitory conditions (Table 1). These results strongly suggest that some carrier-mediated system should contribute to the uptake and transport of D-glucose in the stratified cell layer of human oral mucosa. Both α-methyl-D-glucoside, a specific substrate of SGLT1, and 2-deoxy-D-glucose, a substrate of facilitative transporters, significantly inhibited the transport of D-glucose, but the degree of inhibition was greater with the latter. Similarly, the amount of D-glucose taken up into the mucosal cells reduced more extensively in the presence of 2-deoxy-D-glucose than in the presence of α-methyl-D-glucoside, suggesting that the contribution of the facilitative transporters to D-glucose uptake process is larger than SGLT1. Although this study demonstrated the larger contribution of the facilitative transporters to D-glucose transport across the stratified cell layer, further studies would be needed to clarify how these systems cooperate with each other to transport D-glucose across the stratified cell layer.

The physiological and pathological meanings of the sugar transport systems on the oral mucosa have not been clarified. However, there are specialized transport systems on the oral mucosa not only for sugars but also for other nutrients, such as thiamine (Evered & Mallet 1983) and amino acids (Evered & Vadgama 1981). These systems should function to activate the epithelial cells themselves. Orlando et al (1988) reported that buccal mucosa of humans and experimental animals is capable of active electrolyte transport and that this capacity contributes to the generation of buccal transmural electrical potential difference. This suggests that these transport systems for nutrients might also function to

transport them across the stratified cell layer. Thus, it can be expected that these transport systems are useful for improving the systemic absorption of poorly absorbable drugs via oral mucosa. Our results also suggested that D-glucose is transported across the stratified layer via sugar transporters.

In conclusion, this study using cultured stratified cell layer of human oral mucosal cells clearly showed that the sugar transporters function not only to take up D-glucose into the epithelial cells but also to transport the sugar across the stratified epithelial layer.

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